ANTIVIRAL PROTEINS WITH IMPROVED PROPERTIES AND METHODS THEREFOR

BACKGROUND

Cyanovirin-N (CVN) is an 11 kDa protein (101 amino acids) that has been shown in several studies to have broad anti-viral activity, including anti-HIV activity and anti-influenza virus activities (O'Keefe et al., Antimicrob. Agents Chemo. 47: 2518-2525, 2003). CVN inhibits growth and infectivity of laboratory and primary isolates of HIV. CVN binds to complex mannose structures found on the surface of HIV gp120, and on viral hemagglutinin and on similar structures found in most strains of influenza virus and other enveloped viruses. CVN was initially isolated from cultures of the cyanobacterium *Nostoc ellipsosporum*.

The crystal structure of CVN has recently been determined (Botos et al., Cellular and Molec. Life Sci. 60: 277-287, 2003), showing that the protein exists either as a quasi-symmetric two-domain monomer or as a domain-swapped dimer, explaining the unique mode of high affinity binding of oligosaccharides to CVN. CVN has been expressed in the commensal bacterial species *Streptococcus gordonii*, as a first step in development of a system to deliver and maintain an effective concentration of a microbiocide in the vaginal mucosa (Giomarelli et al., *AIDS* 16: 1351-1356, 2002). While biological activity of CVN for HIV-1 as reported in the literature and reviewed in Botos et al. varies with different methods of assay, in general mutated forms have been found to have diminished or no activity.

A CVN derivative having either increased antiviral properties such as increased affinity for viral glycoprotein, or having decreased toxicity to animal cells, and preferably having both increased affinity and decreased toxicity, would have a greater therapeutic index, and would be highly desirable.

SUMMARY

A feature of the invention provides composition OVT102 having an amino acid sequence as shown in SEQ ID NO: 1.

Another feature provides a composition which is a protein having an amino acid sequence substantially identical to that of wild-type cyanovirin (CVN), and having an additional amino acid sequence X_n at the N-terminal wherein X is an amino acid residue other than cysteine, and n is an integer that is at least 1, wherein the protein has an increased therapeutic index compared to CVN. In a related embodiment of the protein, n is 1. For example, X comprises at least one methionine residue. A therapeutic index is a ratio of a

therapeutic activity to a measure of toxicity. The therapeutic activity of a CVN is an antiviral activity. The composition and protein provided here have increased antiviral activity compared to CVN. Alternatively, the composition or protein provided herein have decreased toxicity to an animal cell, for example, a human cell, compared to CVN. Further, the composition and protein provided herein can have both increased antiviral activity and decreased toxicity. Antiviral activities and toxicity levels of various compositions herein may be measured using animal cells or cell lines that are in standard use for each strain of virus, for example, MDKC (canine kidney cells), or using cultures of established or primary human cell lines, for example, human peripheral blood mononuclear cells (PBMC).

In a related embodiment, the increase in antiviral activity of the protein compared to CVN is at least 10%; for example, the increase in antiviral activity of the protein is at least 20%; is at least 50%; or is at least 100%. The antiviral activity of the protein as determined by standard techniques is reduced infectivity of an enveloped virus. For example, the virus is a retrovirus. The virus is an influenza virus, an immunodeficiency virus, a lymphotropic virus, or a leukemia virus. The immunodeficiency virus is, for example, a virus of a mammalian subject, such as HIV-1, HIV-2, SIV, or FIV. In particular, the immunodeficiency virus is a virus of a human subject, such as HIV-1 or HIV-2.

The increased antiviral activity of the composition or protein can further be attributed to increased affinity for gp120 of HIV. Affinity for gp120 can be determined by any of the assays known to one of skill in the art, for example, by a competition assay comparing relative concentrations of gp120 required to reduce antiviral activity of fixed amount of protein. Affinity of a protein for another molecular entity is generally expressed quantitatively as a constant which is a concentration, such that the lower a constant or concentration would be necessary to cause the protein to bind to the molecular entity to a certain extent, for example, a concentration of a protein necessary to bind to half of a standard amount of an available virus component, the greater the affinity of that protein for the molecular entity such as the virus component.

In alternative embodiments, the enveloped virus is a herpesvirus, a poxvirus, an African Swine Fever virus, a togavirus, a coronavirus, a flavivirus, a paramyxovirus, a rhabdovirus, an arenavirus, or a bunyavirus. The virus is causative of a disease such as influenza, AIDS, Herpes I, Herpes II, hepatitis, smallpox, chicken pox, severe acute respiratory syndrome (SARS), or hemorrhagic fever, such as is caused by Ebola virus.

Yet another embodiment of the invention provides a composition having a protein having an amino acid sequence substantially identical to that of CVN (SEQ ID NO: 2) and

further having at least one additional N-terminal amino acid residue, the protein having enhanced antiviral activity compared to CVN. In this composition, the additional residue can be a hydrophobic amino acid. The term "substantially identical to CVN" means that the composition is at least 70% identical, or at least 80% identical, or is at least 90% identical. The hydrophobic residue is a methionine (M), isoleucine (I), leucine (L), histidine (H), tyrosine (Y), phenylalanine (F), or tryptophan (W). Alternatively, the amino acid is serine (S) or threonine (T). The invention also provides any of these compositions in a pharmaceutically acceptable carrier, or in an effective dose.

In yet another embodiment, the invention provides a kit for anti-viral treatment comprising any of these compositions along with a container and instructions for use. The composition of the kit can be in a pharmaceutically acceptable carrier, or in an effective dose.

In yet another embodiment, the invention provides a nucleic acid encoding any of these compositions or proteins. The invention provides a nucleic acid encoding a gene (Figure 1) for expressing any of these compositions or proteins in a Gram-negative bacterium, for example, the gene has codons optimized for expression in *Escherichia coli.* The invention also provides a nucleic acid comprising a nucleotide sequence as shown in SEQ ID NO: 3, for example, carried on a plasmid or a bacteriophage. Further, the invention provides a method for making an antiviral protein comprising expressing any of these nucleic acids in *E. coli.*

Yet another embodiment of the invention provides a method for treating a subject having an unwanted virus, the method comprising administering to the subject an effective dose of any of the above compositions or proteins. Accordingly, administering the dose is providing a topical medicament. Alternatively, administering the dose is providing a parenteral medicament. Further provided is a method of preventing an unwanted viral infection in a subject, the method comprising administering to the subject any of the above compositions or proteins.

Also provided is a method for removing an unwanted virus from an inanimate object, the method comprising contacting a surface of the object with any of the above compositions or proteins. The inanimate object may be a high value object that has had an opportunity to be contaminated with the unwanted virus, such as a medical device.

Another feature of the invention provides an article of manufacture comprising any of the above compositions or proteins immobilized on a solid substrate. For example, the solid substrate is a planar surface, a bead, a gel, or a fiber. The planar surface comprises a material such as a glass, mica, a metal oxide, or a plastic. The plastic is a polymer such as

polystyrene, polyester, polycarbonate, polyethylene, polypropylene, or a polymer such as nylon.

Yet another feature provided herein is a method for removal of a virus from a bodily fluid, the method comprising contacting the fluid with the article of manufacture above such that the virus remains associated with the article, and further separating the article from the fluid, so that the virus is removed from the fluid. The fluid is blood, serum, lymph, plasma, cerebrospinal fluid, semen, or amniotic fluid.

The invention in another embodiment provides an antibody having affinity and specificity for an epitope comprising the N-terminus of any of the above proteins or compositions. Accordingly, the antibody has an affinity of at least 10^{-8} M.

Another embodiment provides a cell carrying a vector with a nucleotide sequence as shown in SEQ ID NO: 3. For example, the cell is a bacterial cell. For example, the bacterial cell is a species of a genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Sporolactobacillus*, or *Streptomyces*.

Yet another embodiment of the invention herein is a probiotic antiviral medicament for treatment of an epithelial surface of an animal for an unwanted virus, the medicament comprising a cell of the above genera capable of producing spores, which is a *Bacillus*, *Lactobacilius*, or a *Sporolactobacillus*. The medicament is provided in an effective dose. The medicament is provided in a pharmaceutically acceptable carrier or buffer. For embodiments of the medicament as a cell of a *Bacillus* or a *Sporolactobacillus*, the cell can be a stabilized spore preparation. The medicament is used to treat the epithelial surface which is a mucosal surface. For example, the surface is an oral, nasal, rectal, vaginal, or penile epithelium.

A method of preventing or treating an animal epithelium for an unwanted virus is also provided. The method includes administering a probiotic antiviral medicament comprising a lactic acid bacterium capable of expressing any of the above compositions or proteins, for example, capable of expressing OVT 102 having an amino acid sequence as shown in SEQ ID NO: 1. For example, the animal is a human; and the virus is an enveloped virus, such as a retrovirus. The retrovirus is an influenza virus, an immunodeficiency virus, a lymphotropic virus, or a leukemia virus. The immunodeficiency virus is HIV-1, HIV-2, SIV, or FIV. The enveloped virus is a herpesvirus, a poxvirus, an African Swine Fever virus, a togavirus, a coronavirus, a flavivirus, a paramyxovirus, a rhabdovirus, an arenavirus, or a bunyavirus. The virus is causative of a disease such as influenza, AIDS, Herpes I, Herpes II, hepatitis,

smallpox, chicken pox, severe acute respiratory syndrome (SARS), or hemorrhagic fever, for example, as caused by Ebola virus.

Another embodiment of the invention provides a kit for diagnostic detection of an enveloped virus, the kit comprising any of the proteins or compositions provided here, a container, and instructions for use. The kit can further comprise an antibody; the kit can further comprise a detection means such as a colorimetric, fluorescent, bioluminescent or chemiluminescent marker. In yet another embodiment, the invention provides a kit for detection of an enveloped virus, the kit providing OVT102, a container, and instructions for use. The OVT102 in this kit may be immobilized, and detection of the virus may include use of a marker which is colorimetric, fluorescent, chemiluminescent, or bioluminescent. The kit may further include an antibody.

These and further and other objects and features of the invention are apparent in the disclosure, which includes the above and ongoing written specification and the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleotide coding sequence (SEQ ID NO: 3), middle sequence, and a complement of the coding sequence (SEQ ID NO:6), bottom sequence. The nucleotide coding sequence (SEQ ID NO: 3) encodes OVT102 and the encoded OVT102 amino acid sequence (SEQ ID NO: 1) is indicated using the single letter amino acid code, top sequence. The nucleotide sequence was optimized for expression in *Escherichia coli*. The amino acid sequence of the protein produced in *E. coli* starts at the methionine residue (M) encoded by ATG in the nucleotide sequence, and the sequence of the gene to the left of the M has the ribosome recognition site (Shine-Delgarno sequence) and is not translated

Figure 2 shows an alignment of amino acid sequences for the wild type cyanovirin (CVN) (SEQ ID NO:2), top sequence, and OVT102 (SEQ ID NO:1), bottom sequence.

Figure 3 is a graph of codon usage frequency of the gene synthesized to encode OVT102 using codon optimization.

Figure 4 is a complete sequence (SEQ ID NO: 4) of plasmid pET27b carrying the gene (SEQ ID NO: 3) encoding OVT102 having codons optimized for *E coli* expression (plasmid pET27b-OVT102), with the amino acid sequence as shown in Figure 1 (SEQ ID NO: 1). The OVT102 gene is highlighted in light gray, and the start and stop codons in dark gray.

Figure 5 is a drawing of a map of expression plasmid pET27b-OVT102, showing the locations of genes and significant restriction sites.

Figure 6 is a photograph of a gel electrophoretogram (SDS-PAGE) analysis of bacterial cell lysates from separate production fermentors containing *E. coli* strain BL21:OVT102. Lane 1 (left most) contains molecular weight standards. Increasing amounts of purified OVT102 that serve as reference standards are shown in lanes 2-5. Lanes 6-12 contain samples of whole cell lysates from six different fermentations of the strain, showing that a band co-migrating with pure OVT102 was found in all lysates.

Figure 7 is a reverse phase high pressure ion exchange liquid chromatogram showing a purified sample of OVT102. The trace labeled A shows the absorbance at wavelength 280 nm (A_{280}), and coincides with OVT102 activity. The second trace marked B shows the salt gradient (as measured by the change in solution conductivity (mS/cm) of the elution buffer.

DETAILED DESCRIPTION OF EMBODIMENTS

Certain extracts of cultured cyanobacteria (previously known as blue-green algae) have been found to produce proteins with antiviral activity, including activity against human immunodeficiency virus (anti-HIV activity; U.S. patents 5,821,081, 5,843,882, and 5,962,653) and related strains such as HIV-1, HIV-2 and SIV. The material known as cyanovirin (CVN) was isolated from *Nostoc ellipsosporum*. These references show expression of CVN, a 101 amino acid protein, with additional amino acids at the N-terminal sequence ("the FLAG" sequence DYKDDDDK, SEQ ID NO: 5) to create a DYKDDDDK-CVN fusion protein for purposes of purification. These references also show that HIV gp120 is a principal molecular target of CVN, and that CVN has activity against a broad range of clinical isolates, suggesting that the naturally occurring drug-resistant mutants of this virus remain susceptible to CVN anti-viral activity. Antibody to CVN was produced in rabbits (U.S. patent 5,998,587).

CVN has been described for use as an anti-viral therapeutic agent (U.S. patent 6,015,876) and virucidal agent. Immunization with CVN to elicit anti-epitope antibodies and create an image of the target, gp120 is described in U.S. patent 6,193,982. Further, a genetic fusion of CVN to viral glycoprotein was described in U.S. patent 6,245,737. Further, CVN has been suggested as an agent for immobilization to an affinity matrix, for removal of immunodeficiency viruses from a variety of materials, including body fluids (U.S. patent 6,428,790). Each of U.S. patents 5,821,081, 5,843,882; 5,962,653; 5,998,587; 6,015,876; 6,193,982; 6,245,737 and 6,428,790 is incorporated herein by reference in its entirety.

Applications of CVN for the useful purposes described in these references would be substantially improved by use of a novel form having increased affinity for those viral targets described, having affinity for additional viral targets, and having decreased toxicity for animal cells, particularly human cells.

Construction of gene encoding OVT102 and a vector for high level expression

A process was developed to construct a new CVN gene and expression plasmid, for expression of the protein in *E. coli*, to introduce this new expression plasmid into an appropriate production strain of *E. coli*, to isolate a production strain that yields maximal protein, and to characterize the properties of this protein. Because by this process the protein was being expressed in bacteria, a start codon encoding methionine was included (see Figure 2). This form of CVN is referred to as OVT102.

For efficient expression of CVN in *E. coli*, a gene encoding CVN was synthesized (Figure 1) and cloned into an appropriate, high-level bacterial expression vector (Figures 4 and 5). The gene was designed to contain those codons (see Figure 3) that are preferentially utilized by the production organism, *E. coli*. In addition to selecting the codons for optimizing of expression, further aspects of design of the gene were made to achieve highest possible protein production.

The amino acid sequence and a newly designed nucleotide sequence of the gene encoding this amino acid sequence are shown in Figure 1. The alignment of amino acids for OVT102 and CVN is identical after the initial methionine residue of OVT102, as shown in Figure 2.

Figure 3 shows the codon frequencies of the OVT102 gene sequence. Codon usage in the natural CVN gene is similar to that of the gene designed to be expressed in *E. coli*, as CVN is a product of the prokaryote cyanobacterium *Nostoc ellipsosporum*.

The codon-optimized gene was inserted into bacterial expression vector pET27b (Stratagene, Inc., La Jolla, CA) that provides high expression levels in *E. coli*. The complete sequence of plasmid pET27b-OVT102 is shown in Figure 4. A map of the plasmid showing all the functional and regulatory elements is shown in Figure 5. Production is under the control of a bacteriophage T7 promoter, which has been engineered to be controlled by the Lac operator and repressor, so that production is inducible by addition of the galactoside analog isopropyl-β-D-thiogalactopyranoside (IPTG). The vector contains a kanamycin resistance gene for selection of clones successfully transformed with the construct, and for maintenance of the vector by applying selective pressure on cells during fermentation. The gene is inserted as an *Nhe*I-*Nde*I fragment, and contains both an ATG start codon and a TAA

stop codon. The expression plasmid parental vector was chosen to contain no leader sequences or affinity tags for the cloned insert, such that OVT102 protein expressed in the host cells was designed to appear intracellularly in the form of inclusion bodies. From this expression, OVT102 would be readily isolated away from other cellular components, chemically treated to denature, and then be refolded into active protein.

Plasmid pET27b-OVT102 was introduced into host organism *E. coli* strain BL21(DE3), by electroporation using a BioRad Gene Pulser, and kanamycin resistant colonies were selected. The presence of the gene encoding OVT102 in plasmid DNA isolated from five individual colonies was confirmed by sequencing of both strands of the gene. One of the confirmed colonies was chosen as a reference strain, was amplified by growth in rich growth media, and cells mixed with sterile glycerol to obtain stock cultures, and frozen for future use as inocula for culture. This strain is called *E. coli* BL21(DE3):OVT 102.

To produce initial material for protein characterization, a standard unoptimized fermentation protocol developed for production of proteins in inclusion bodies in *E. coli* was implemented. *E. coli* BL21(DE3):OVT102 was grown to saturation in a 2L shake flask at 37°C and induced with IPTG.

A gel showing expression of the OVT102 protein by cells in each of a number of different shake flask experiments is shown in Figure 6. A band of protein was observed at a molecular weight of about 11kD in cells of each of six independent shake flask batches. This mobility is predicted molecular weight of the CVN, the same as that of pure OVT102 shown in lanes 2-5. Amino acid analysis of the purified OVT102 indicates that the observed ratios of amino acids were substantially identical to those predicted based on the nucleotide sequence of the gene.

Optimization of cell growth, harvesting and protein purification

To obtain a large quantity of OVT102, fermentation and solubilization of proteins from inclusion bodies were performed as described herein. *E. coli* cells of strain BL21(DE3):OVT 102 were grown in a bench top fermenter, and expression of OVT102 protein was induced by addition of 1mM IPTG. Growth was continued for 6 additional hours in the fermentor. Cells were harvested, ruptured, and inclusion bodies were obtained and treated as follows.

Inclusion bodies were harvested and washed three times with water by centrifugation. A final pool of inclusion bodies as a loose pellet was solubilized in 8M urea, 80mM TRIS pH8.5 and 5mM EDTA. The pellet was resuspended in this buffer by vigorous mixing.

OVT102 was refolded from urea-solubilized inclusion bodies obtained from fermentation by dilution into 2.66mM oxidized glutathione in water. The refolded product was clarified by filtration through a 0.45 micron filter and the protein was purified as described herein. Total protein content was determined by measuring absoprtion of light at 280 nm (A_{280}).

To purify the protein, adsorption onto a first chromatographic medium (primary capture) was performed using QAE550C (Tosoh BioBiosciences, Montgomeryville, PA). Protein was adsorbed to the QAE550 resin in 20mM TRIS pH 9, and eluted with a salt gradient (0 to 400 mM NaCl), and peak fractions were pooled. Analytical data (Tosoh) showed that the three leading peaks from the Q Sepharose chromatography column were high purity OVT102.

Secondary capture and purification were performed by loading the pooled OVT102 fractions from primary capture onto a column containing Phenyl Sepharose 6 FF (High Sub.; Amersham Biosciences). OVT102 was eluted with a gradient of ammonium sulfate in 50mM TRIS pH 8. Additional purification was performed with QHP anion exchange medium (Amersham, Piscataway, NJ)) and filtration through a Hydrosart (Sartosius, Goettingen, Germany) membrane having a molecular weight cutoff of 5,000 daltons.

Analysis of the product was achieved by high pressure ion exchange chromatography, ELISA and SDS-PAGE (Figure 6). The ion exchange chromatogram (Figure 7)indicates that the material was substantially pure based on total peak area.

While the invention has been described with reference to specific embodiments, modifications and variations of the invention may be constructed without departing from the scope of the invention. Additional embodiments are shown in the Examples and in the claims, which should not be construed as further limiting.

EXAMPLES

The following materials and methods were used throughout the Examples herein.

Media and buffers

Bacterial cells were grown in a conventional bacterial soy-based medium in shake flask cultures which were used to inoculate a Braun Biostat U fermenter. High volume fermentation of cells was performed at 37°C, pH 6.8 in medium containing yeast extract. Production of OVT102 was induced with IPTG. Buffers used for purification of OVT102 are described in the specific examples.

Complete tissue culture medium was RPMI 1640 with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, and 100 micrograms/ml streptomycin. Infection medium was DMEM with 10% bovine serum albumin, glutamine penicillin and streptomycin as above, 0.1mM NEAA (Cambrex, Hopkinton, MA), 1 mM sodium pyruvate, and 1 microgram/ml TPCK-treated trypsin (New England Biolabs, Beverly, MA).

Assay of anti-viral and toxicity activities

Viruses were obtained from American Type Culture Collection (Manassas, VA), and were assayed using MDCK cells (for assay of influenza virus) at $5x10^4$/ ml or fresh human PBMCs (for HIV-1; PBMCs obtained from Interstate Blood Bank, Inc., Memphis TN).

For influenza assays, MDCK were added to each well of 96-well flat bottom tissue culture plates in a volume of 100 microliters/well. One day following plating of cells, microtiter plates were aspirated to remove medium, the monolayer was washed with phosphate-buffered saline (PBS) and samples of each of the diluted viruses and test compound drugs diluted in infection medium were added to each well in triplicate, each virus and compound in a volume of 100 microliters. Each plate included control wells (cells only), virus control wells (cells plus virus), drug toxicity control wells (cells plus drug), drug colorimetric control wells (drug only), and experimental wells (drug plus cells plus virus).

After 6 days of infection at 37°C in a 5% CO_2 incubator, test plates were analyzed by incubating with tetrazolium dye XTT-PMS (2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2*H*-tetrazolium- 5-carboxanilide; Molecular Probes, Inc.) reagents for 4 hours under the same incubator conditions, and were analyzed by absorbance of the reduced XTT-PMS at 450 nm in a Molecular Devices microplate reader. XTT was prepared daily as a stock of 1 mg/ml in culture medium. Phenazine methosulfate (PMS) was prepared at 15 mg/ml in PBSA and stored frozen in the dark. XTT/PMS was prepared immediately prior to use by adding 40 microliters of PMS per ml of XTT, and was added to each well in a volume of 50 microliters. IC_{50} (50% inhibition of virus replication), and TC_{50} (50% reduction in cell viability) and therapeutic index (TI: IC_{50}/TC_{50}) were calculated. A four hour incubation was determined empirically to yield a linear response for XTT dye reduction with the number of cells used per well.

For HIV-1 assay, leukophoresed blood cells were washed with Dulbecco's phosphate buffered saline (PBS), and diluted 1:1 with PBS, and were layered on Ficoll-Hypaque density gradient in a 50 mL conical centrifuge tube and centrifuged 30 min at 600g. Banded PBMCs were gently aspirated from the interface and washed three times with pBS and enumerated by trypan blue exclusion. Cells were resuspended at $1x10^6$/mL in RPMI 1640 with 15% Fetal

Bovine Serum, 2 micrograms/mL PHA-P, 2mM L-glutamine, 2 micrograms/mL PHA-B, 100 Units/mL penicillin, and 100 micrograms/mL streptomycin and incubated for 48-72h at 37°C. PBMCs were centrifuged and resuspended in tissue culture medium (as above without the PHA-P and with addition of and 3.6 ng/m recombinant human IL-2). Cultures were maintained until use by change in culture medium (1/2 volume) every 3 days. Most assays used cells that had been induced to proliferate for about 72 hours.

For the HIV-1 assay, PHA-P stimulated PBMCs from three donors were pooled to minimize individual variation, and were resuspended in fresh tissue culture medium at $1x10^6$/mL and a volume of 50 microliters of cell suspension was plated in wells of a 96-well round-bottom microplate. Compounds in the medium in volumes of 100 microliters were transferred to the wells and infected with virus at a predetermined dilution (using 4-fold greater concentration than final concentration). About 50-150 time TCID50 of each virus was added per well (final multiplicity of infection, MOI=0.05-0.10), in triplicate, and plates were cultured for 7 days. HIV-1 replication was quantified by measurement of reverse transcriptase activity. Separate plates were identically prepared without virus for drug cytotoxicity studies with XTT as described.

Microsoft Excell 2000 was used to analyze and graph data. Values for IC_{50} and TC_{50} were expressed as mean $\pm$ standard deviation for the triplicate assay results. Ribavirin was used a a positive control antiviral agent for influenza assays, and AZT was used as a positive control antiviral agent for HIV assays.

Reverse transcriptase activity assay

Reverse transcriptase was measured in cell-free supernatants using a radioactive incorporation polymerization assay. Tritiated thymidine triphosphate (TTP at 1 Ci/mL,; New England Nuclear Corp.) was used at 1 microliter per reaction. poly rA and oligo dT were prepared at 0.5 mg/mL and 1.7 Units/mL, respectively, from stock solutions kept frozen.RT reaction buffer was prepared fresh daily consisting of: 125 microliters of 1 M EGTA, 125 microliters distilled water (dH_2O), 125 microliters of 20% Triton X-100, 50 microliters 1M Tris ph 7.4, 50 microliters of 1M dithiothreitol, and 40 microliters of 1M $MgCl_2$. For each reaction, 1 microliter TTP, 4 microliters dH_2O, 2.5 microliters of rA and 2.5 microliters of of reaction buffer were mixed. Ten microliters of this total reaction mixture was placed in a well of a 96 well micro-titer plate and 15 microliters of virus-containing cell culture supernatant was added and the contents were mixed. The plate was incubated at 37°C in a humidified incubator for 90 min. At the end of this reaction time, 10 microliters of the reaction was spotted onto a DEAE filter mat in an appropriate plate format, washed 5 times

for 5 min each was in 5% sodium phosphate buffer, 2 times for 1 min each in distilled water, 2 times for 1 min each in 70% ethanol, and then air dried. The dried filtermate was placed in a plastic sleeve, and 4 mL Opti-Fluor O was added to each sleeve. Incorporated radioactivity was quantified using a Wallac 1450 Microbeta Trilux liquid scintillation counter.

Example 1. Characterization of OVT102

The amino acid residue at the N-terminal sequence of OVT102 was determined to be methionine, consistent with the intent of the gene construction, and this variant of CVN is termed OVT102. To perform N-terminal analysis, the peak fraction from QHP anion exchange chromatography, which contained highly purified OVT102, was used.

SDS PAGE analysis of fractions from purification steps was performed, as was western blotting and the N-terminal sequence determination. The N terminal sequencing procedure obtained amino acid sequence MLGKFSQTCY (SEQ ID NO:7), confirming a correct sequence of that predicted for OVT102, a CVN variant having an additional N terminal methionine.

Example 2. OVT102 has greater antiviral activity than CVN for HIV strains

For HIV isolates, OVT102 showed an IC_{50} (i.e., concentration at which 50% of viral replication was inhibited) of 2 to 28 nM, which surprisingly is a greater activity than that of CVN in in vitro infectivity assays. In these same assays, the IC_{50} for CVN was consistently greater, i.e., for five different HIV isolates, IC_{50} of OVT102 inhibition of HIV infection of freshly isolated human peripheral blood mononuclear cells ranged from 1.8 to 4.2-fold lower than that of CVN, although in a few HIV strains the IC_{50} of these compounds was similar. OVT102 also inhibited HIV attachment to viral entry co-receptors CXCR4 and CCR5 with an IC_{50} of 29 to 900 pM.

Compounds were tested against five strains of each of influenza A (A1/Denver/1/57, A/NWS/33, A/WS/33/, A/Hong Kong/8/68, and A/PR/8/34) and influenza B (B/Taiwan/2/62, B/Mass/3/66, B/MD/1/59, B/Lee/40, and B/Hong Kong/5/72). The data showed that for two different batches of OVT102, antiviral activity IC_{50} values against influenza A ranged from 1.0 to 20 nM and 4.0 to 60 nM for influenza B. Inhibition of influenza virus was found for IC_{50} values ranging from 1 to 10 for strains of most influenza viruses, including types A and B, and including a neuraminidase-inhibitor resistant strain. These data indicate that for many influenza strains, the IC_{50} values are at least as good as those found for CVN in independent assays. In light of the decreased toxicity of OVT102 compared to CVN (see Example

below), it is likely that the therapeutic index of OVT102 will be significantly greater for this molecule for influenza and other viruses.

Example 3. OVT102 has greater affinity for gp120 of HIV-1 than CVN

A gp120 ELISA assay was performed to compare relative binding by native CVN isolated from cyanobacteria with binding by OVT102. The assay was performed in wells of a 96 well microtiter plate, by coating each well with 50ng gp120 and blocking with 0.1% bovine serum albumin. The gp120 and blocking solutions were removed and calibration standards, test samples, or blank (negative controls) were added.

Test samples were native CVN or OVT102 at dilutions ranging from 1-10 ng/ml based on A_{280} protein concentration determinations. After incubation and rinsing, wells were treated with rabbit anti-CVN antibody at 1:3000 dilution, followed by Alkaline Phosphatase-labelled goat anti-rabbit antibody. Alkaline phosphatase substrate was added, and the binding level was determined by measuring absorbance at 405nm. The absorbance at 405 nm is a measure of alkaling phosphatase substrate hydrolyzed, and is directly related to the amount of CVN or OCT102 bound to gp120.

Based on an average of twelve determinations, the binding of OVT102 to gp120 was 1.4-fold greater than the binding of native CVN to gp120.

Example 4. OVT102 has reduced toxicity compared to CVN

Toxicity data using human PBMCs show TC_{50} values that indicate that OVT102 is surprisingly less toxic than CVN. A titration of percent of cell control as a function of concentration yielded a TC_{50} value for CVN of 320 nM, and for OVT102 of 524 nM, i.e., a greater amount of the latter is required to kill 50% of the cells, based on the XTT assay described above. When inhibition of HIV-1 replication and cell inhibition are measured simultaneously, the increase in antiviral effectiveness and decrease in toxicity gave an increase in therapeutic index of OVT-102 of greater than four-fold that of CVN, for example using HIV-1 clade B (BR/92/014).

The therapeutic index (TI) values range from >3.9 to 30 for OVT102 against influenza A, values of >1.6 to >7.5 against influenza B. These values are more favorable than that of CVN, which ranged from >11.3 to >230.8 for influenza A, and >1.6 to >45.5 for influenza B.

The consistent greater effectiveness of OVT-102 and decreased toxicity compared to CVN against several strains of each of HIV-1 and influenza viruses indicate that this molecule has desirable properties as an anti-viral agent for enveloped viruses